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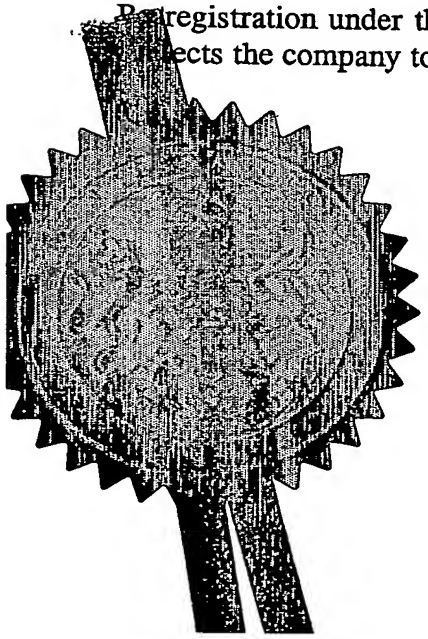

Signed P. Mahoney

Dated 3 October 2003

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2. Pat 0220619.1
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 8459331001

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If the applicant is a corporate body, give the country/state of its incorporation 6026322001

4. Title of the invention

METAL BINDING PRECURSORS FOR THE SYNTHESIS OF PEPTIDE-METAL CONJUGATES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

N/A

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43-45 BLOOMSBURY SQ.
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WC1A 2RA

Patents ADP number *(if you know it)*

5117
49/30m.

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
N/A		

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

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Description	5
Claim(s)	1
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Priority documents	N/A
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

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12. Name and daytime telephone number of person to contact in the United Kingdom DR. PHILIP J. BLOWER
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Metal binding precursors for the synthesis of peptide-metal conjugates

This invention relates to precursors for use in the development and synthesis of radiolabelled biological molecules, in particular synthetic peptides, in which the radiolabel is a metallic radionuclide. The radiolabelled molecules are for use in medical diagnosis by single photon emission imaging or positron emission tomographic imaging, or in targeted radionuclide therapy.

Direct imaging of a variety of biochemical processes is now practicable through the development of radiopharmaceuticals targeted towards specific disease-associated molecular targets. This was made possible by the new discoveries in the field of disease-related changes in cellular communication and metabolism, especially in cancer. To support these new diagnostic applications, methods for linking radioisotopes to the appropriate targeting biomolecules were required to replace the simple metal chelates and ions used previously. In the 1980s and 1990s methods were developed for labelling biomolecules, especially monoclonal antibodies, with radionuclides such as technetium-99m and indium-111. In its most developed form, this typically entailed covalent attachment of a bifunctional chelator to a protein, followed by labelling with the radiometal, or even synthesis of a radiometal·bifunctional chelate which was subsequently attached to the protein. As recognition grew in the 1980s that monoclonal antibodies are too large to offer ideal pharmacokinetics, focus shifted onto smaller molecules such as antibody fragments and especially smaller peptides targeted towards receptors present in lesions such as tumours and thrombi. Radiolabelled octreotide, a small peptide that binds to somatostatin receptors expressed in many cancers, is the leading example but several others (e.g. vasoactive intestinal peptide, bombesin etc.) are now finding widespread clinical use.

Although the transition to smaller molecules brought with it the opportunity to use peptides produced by solid phase peptide synthesis (SPPS) rather than proteins of biological origin, the same methods were used to label them as had been used to label antibodies. These methods have several disadvantages, which are more problematic with small peptides than with large proteins. The most suitable sites for attachment of a bifunctional chelator in most peptides are the ε-amino groups of lysine residues and the N-terminus, because they are very reactive nucleophiles and form very unreactive covalent links with the chelator. If there is more than one lysine, the site of modification becomes uncertain. For instance, if the peptide has two lysines, together with the N-terminus these will present three possible sites for conjugation, hence forming as many as eight products when treated with an active-ester-containing bifunctional chelator or radiolabelled bifunctional chelate. Each of these products will have a different biodistribution and different affinities for the target (some of them may have lost all target affinity) and such a mixture is not acceptable for clinical use. Moreover, one or more of the lysines may be essential to the biological activity of the peptide. A simple solution has been to incorporate the chelator, or a radiolabelled chelate or organic prosthetic group, as the last step of SPPS. This, however, has the limitation that the chelator has to be at one end of the peptide chain, which is frequently essential to the biological activity of the peptide.

The state of the art in linking radiometals to peptides encompasses a number of approaches. Some have the advantage of incorporating the metal binding sequence during SPPS, and others have the advantage of incorporating chelators that are specifically designed for the particular metal. None, however, have both of these advantages. For example, technetium-chelating amino

acid sequences such as gly-gly-cys are incorporated during SPPS or recombinant protein production, but this sequence is not ideal for its purpose, and merely represents the best that can be achieved for chelating the TcO^{3+} core using "standard" amino acids (i.e. those coded through tRNAs). Likewise, polyhistidine sequences such as hexahistidine can be incorporated during SPPS, but again they merely represent the best sequence of *coded* amino acids achievable for chelating the $Tc(CO)_3^+$ core. Conversely, the synthetic technetium ligand hynic (hydrazinonicotinamide) probably represents the most convenient and efficient labelling system to date for use with Tc-99m, but it has so far only been used by conjugating it to a pre-formed peptide, with all the associated problems outlined above. An alternative that offers convenience of labelling is the "direct labelling" method in which antibodies and peptides containing disulfide bonds can be reduced and labelled with Tc-99m or Re-188. However, the chemistry of these methods is poorly understood, and there are major stability and biological activity problems as demonstrated by the work of several groups world wide with antibodies and somatostatin analogues.

According to the present invention, there is provided a more versatile and controlled approach to the synthesis of peptide conjugates for radiolabelling. In this invention, metal-chelating precursors, designed to bind specific metallic radionuclides and incorporating a pendant protected (e.g. Fmoc) amino acid functionality, are synthesised. This allows an advance on conventional synthesis of peptide-chelator conjugates in that the chelator is attached to an amino acid before rather than after SPSS assembly of the peptide chain, with the advantages identified below.

Preferably the metal binding functional group will be specifically designed to form a kinetically stable complex with a suitable metallic gamma- or positron-emitting radionuclide for diagnostic imaging (including but not limited to technetium-99m, indium-111, copper radioisotopes, and lanthanide radioisotopes) or a particle-emitting metallic radionuclides for radionuclide therapy (including but not limited to rhenium-186, rhenium-188, copper-64, copper-67, and lanthanide radioisotopes). Suitable chelating or metal binding groups may preferably be chosen from several structures including but not limited to the hydrazinonicotinamide group, di- or poly-thiol groups, macrocyclic ligands incorporating amine, thioether, or phosphine donor groups, or polyaminocarboxylate groups.

The amino acid functionality may preferably but not necessarily be derived from an enantiomerically pure amino acid with an amine-containing side chain, such as L-lysine or L-ornithine or a similar homologue.

These chelating amino acids are used in protected form as amino acid building blocks and can be inserted into a synthetic peptide sequence during SPPS at any predetermined position in the sequence, in place of lysine or any other amino acid, or in addition to native amino acids. These advantages overcome the problems associated with conventional methods described above. They are particularly suited to development of combinatorial libraries of radiolabelled peptides, which will be especially significant in the development of peptide radiopharmaceuticals targeted towards the many new cancer-related targets likely to be identified in the near future through developments in proteomics.

The advantages of the chelating amino acid method over conventional post-SPPS conjugation may be summarised as follows:

1. The method is versatile and flexible: it incorporates the chelator site-specifically *anywhere* in sequence, not just terminally or at one or more lysines.
2. It can leave lysines unmodified.
3. It is economic since no post-SPPS modification is needed.
4. It is suitable for a combinatorial approach to generate libraries of peptide radiopharmaceuticals from which imaging agents for specific targets can be selected.

The advantages of the chelating amino acid method over conventional chelating amino acid sequences (such as gly-gly-cys or poly-his, which are at present the only chelating structures that are incorporated *during* SPPS) may be summarised as follows:

1. It is versatile and flexible, in that it can be used for any metal since a specifically designed synthetic chelator can be used, and there is no need to rely on coded amino acids which are not ideal chelators.
2. It gives total control over the labelling site.

Figure 1 shows a scheme describing the synthesis of such a chelating amino acid building block together with its structure (molecule 1). Molecule 1 incorporates an amino group in Fmoc protected form (labelled **1**), a carboxylic acid group (labelled **2**) and the technetium-binding group hynic in Boc protected form (labelled **3**).

Figure 2 shows, by way of example, the structure of a synthetic peptide, molecule 2, prepared using molecule 1, in which the hydrazide group is protected by a trifluoroacetate group.

Figure 3 shows, by way of example, the probable structure insofar as can be established by the available spectroscopic data and by comparison with the literature, of a radiolabelled derivative of molecule 2.

Specific embodiments of the invention will now be described by way of examples.

Example 1

Synthesis of Fmoc-lysine-hynic-Boc (molecule 1, see Fig. 1). The synthetic route is summarised schematically in Figure 1. To the N-hydroxysuccinimide ester of hynic-Boc (1.79mmol), prepared by a literature method, in ~10mL dimethylsulfoxide is added 220mg (0.597mmol) of N-α-Fmoc-Lys (purchased commercially). An additional 5mL of dimethylsulfoxide is added to aid solubility and the mixture is stirred overnight at room temperature. The mixture becomes a clear yellow solution after 60 minutes, then becomes cloudy after 180 minutes. This reaction mixture is added slowly to 60mL of H_2O with stirring, after which the pH is 4. The mixture is stirred overnight at room temperature. The crude precipitated product is filtered off and washed with 50mL H_2O and dried under vacuum to give 460mg of an off-white solid. The crude product is dissolved in methanol and purified by normal phase HPLC using isocratic elution with methanol. The broad second peak is collected and dried under vacuum. The product is an off white-yellow solid, 229mg, 64% yield relative to N-α-Fmoc-Lys. Analysis by reversed phase HPLC (RP-HPLC) and electrospray mass spectrometry (ES-MS) gives a major peak at 26.91 minutes with m/z (relative abundance) shown in the table below:

Charged species assigned	Observed m/z	Calculated m/z
[N-α-Fmoc-N-ε-(**Hynic-Boc**)-Lys+H]$^+$	604.1 (100)	604 (100)
[(N-α-Fmoc-N-ε-(**Hynic-Boc**)-Lys)$_2$+H]$^+$	1207.1 (16)	1207 (100)
[N-α-Fmoc-N-ε-(**Hynic-CO_2H**)-Lys+H]$^+$	548.1 (53)	548 (100)
[N-α-Fmoc-N-ε-(**Hynic**)-Lys+H]$^+$	504.3 (30)	504 (100)

NMR and IR spectra and elemental analysis are consistent with the proposed product.

Example 2

Synthesis of trifluoroacetate-protected hynic-modified salmon calcitonin (molecule 2). The peptide is synthesised using standard Fmoc SPPS with the following modifications. N-α-Fmoc-N-ε-(Hynic-Boc)-Lys (molecule 1) is used in place of N-α-Fmoc-Lys at position 18 of native salmon calcitonin. All amino aids are used at x10 excess except for molecule 1, which is used at x5 molar excess (30.2 mg). The completed 32-amino acid sequence is cleaved from the resin by treatment with trifluoroacetic acid for 3 hours. After the precipitation with diethyl ether the product is reconstituted with 2.5mL H_2O. Analytical RP HPLC ES-MS shows two major product peaks: reduced sCtLys^{18}-Hynic and reduced sCtLys^{18}-Hynic-TFA (in which the hydrazide is protected with a trifluoroacetate group) identified by ES-MS. These two fractions are purified by reversed phase HPLC. The intramolecular disulfide bond is formed by aerial oxidation in 15mL of helium-degassed 0.1M sodium bicarbonate pH8.2. RP-HPLC and ES-MS show that the non-protected hydrazine groups are vulnerable to N-N bond cleavage under these conditions, while the TFA protection prevent this.

The oxidised product sCtLys^{18}-Hynic-TFA is purified by preparative RP HPLC, frozen in liquid nitrogen and then freeze-dried. Post-disulfide bond oxidation, analytical RP HPLC ES-MS of the target product peak is consistent with m/z (relative abundance) shown in the table below:

Charged species	Observed m/z	Calculated m/z
{$C_{153}H_{244}F_3N_{47}O_{50}S_2$ Average MW 3663.1}		
[sCtLys^{18}-Hynic-TFA+2H]$^{2+}$	1832.5 (100)	1832.6
[sCtLys^{18}-Hynic-TFA+3H]$^{3+}$	1221.9 (34)	1222.0

Example 3

Synthesis of hynic-modified salmon calcitonin labelled with technetium-99m/tricine (Molecule 3, Figure 3), and its affinity for human calcitonin receptors. Prior to radiolabelling, the TFA protecting group is removed, as follows: To 100μL of sCtLys^{18}-Hynic-TFA (0.275mg/mL) in 10mM sodium bicarbonate pH8.2, is added 200μL of 0.1% TFA in H_2O. The mixture is left at room temperature for 6.75 h, pH2.5. A 21.8μL aliquot containing 2.0μg of peptide is treated with 50μL of tricine (100mg/mL in H_2O pH3.5), 5μL of $SnCl_2$ (0.2mg/mL in H_2O) and 100μL of $Na^{99m}TcO_4$ (250MBq) in physiological saline. The mixture is left at room temperature for 60 minutes. Analysis of the product by radiochromatography (instant thin layer chromatography and reversed phase HPLC) shows that 92-96% or the radioactivity is eluted in a single peak corresponding to labelled peptide, with the remainder eluting as pertechnetate. After incubation in human serum for 30 min, reversed phase HPLC and size exclusion chromatography show that the product is stable with no significant pertechnetate formation or binding of radioactivity to serum proteins. A triplicate calcitonin receptor binding assay with MCF-7 human breast cancer

cells shows strong, specific receptor binding of the radiolabelled: at a concentration of 1nM in a multi-well plate, the labelled peptide gives 23638 (SEM 490) counts per minute bound to MCF7 cells, 941 counts per minute bound to the well in the absence of cells, 726 counts per minute bound to cells in the presence of 1μM cold salmon calcitonin, and 588 counts per minute bound to wells in the absence of cells but in the presence of 1μM cold salmon calcitonin.

CLAIMS

1. A chelator-derivatised amino acid into which three functionalities are incorporated: 1) a primary or secondary amino group, which may be protected for the purposes of solid phase peptide synthesis using standard protecting groups including but not limited to Fmoc; 2) a carboxylic acid group; 3) a chelating or metal-binding group designed for the specific, kinetically stable attachment of the metallic radionuclide chosen for diagnostic imaging or treatment.

2. A chelator-derivatised amino acid as claimed in Claim 1, wherein the metal binding group is hynic (hydrazinonicotinamide)

3. A chelator-derivatised amino acid as claimed in Claims 1 and 2, wherein the amine and carboxylic acid groups are embodied in the amino acid L-lysine (as in molecule 1 in Figure 1 of the accompanying drawings) or its homologues including but not limited to L-ornithine.

4. A chelator-derivatised amino acid as claimed in Claims 2 and 3 wherein the hydrazine group is protected by a suitable protecting group such as Boc as illustrated as molecule 1 in Figure 1 of the accompanying drawings) for the purposes of solid phase peptide synthesis, or trifluoroacetate (as in molecule 2 in Figure 2) for the purposes of preventing cleavage of the N-N bond during subsequent manipulation of the peptide.

5. A method of synthesising a peptide modified with a chelator as claimed in Claims 1, 2 and 3, in which the chelator is incorporated at any pre-determined position in the sequence during conventional solid phase peptide synthesis, by using chelator-derivatised amino acids such as those described in Claims 1, 2 and 3 as the amino acid building blocks.

6. A derivative of salmon calcitonin (molecule 3) in which lysine-18 is replaced by a hynic derivative as shown in Figure 3, and which is thus suitable, after the necessary deprotection, for convenient radiolabelling with technetium isotopes for purposes of diagnostic imaging of disease sites in which receptors are abundant.

ABSTRACT

Metal binding precursors for the synthesis of peptide-metal conjugates

Specific metal-chelating precursors incorporating a pendant protected (e.g. with Fmoc) amino acid functionality are synthesised. The pendant amino acid functionality allows the chelator to be inserted into a synthetic peptide sequence during standard solid-phase peptide synthesis at any predetermined position in the sequence, in place of lysine or any other amino acid, or in addition to native amino acids. An example is a conjugate incorporating Fmoc-protected L-lysine and the technetium-binding group hynic (hydrazinonicotinamide), shown as molecule 1 in Figure 1 of the accompanying drawings. These molecules permit synthetic approaches with greater flexibility and control of the site of labelling than conventional methods. They are particularly suited to development of combinatorial libraries of radiolabelled peptides, which will be especially significant in the development of radiopharmaceuticals targeted towards the many new cancer-related targets likely to be identified in the near future through developments in proteomics.

Figure 1 to accompany abstract

DRAWINGS

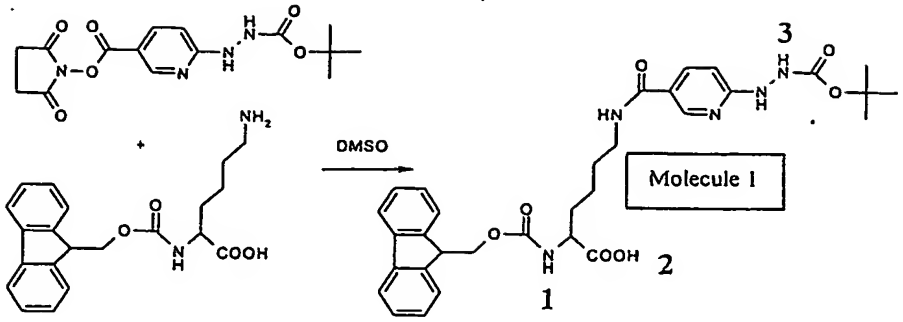

Figure 1

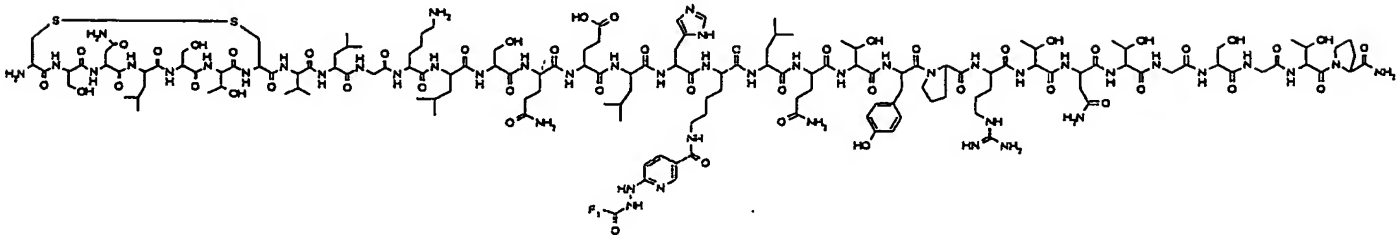

Figure 2. Molecule 2

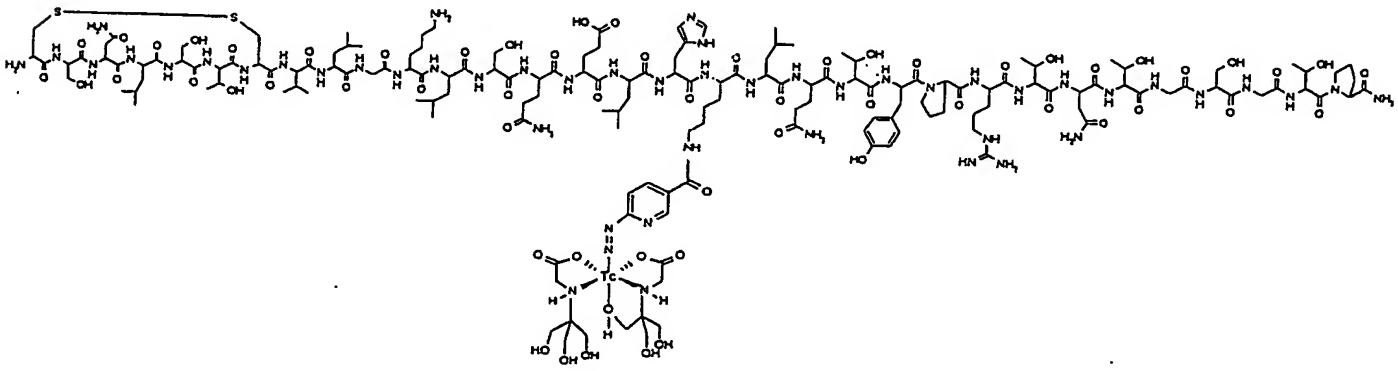

Figure 3. Molecule 3